Proposed OPPTS Science Policy: PPARα-Mediated Hepatocarcinogenesis in Rodents and Relevance to Human Health Risk Assessments

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PREFACE

The U.S. Environmental Protection Agency (EPA) uses two assumptions concerning the assessment of laboratory animal tumors (USEPA, 1999; 2003). The first is that the tumor findings in the experimental animals are relevant to the assessment of potential cancer hazards and risks in humans. The second is that, if the animal evidence is sufficient to support a conclusion that a carcinogenic response has occurred in the test species, and the mode of action (mechanistic) information on the way(s) a chemical may induce tumors either is absent or fails to support a non-linear dose response, a linear dose-response extrapolation is used to estimate risks at environmental exposure levels. Each of these assumptions is rebuttable in the face of convincing scientific information.

EPA has issued general guidance on the use of animal and other data to assess the human carcinogenic potential of environmental agents (USEPA, 1986) and has proposed updates to that guidance more recently (USEPA, 1996; USEPA, 1999; USEPA, 2003). To date, EPA has also developed science policies on the interpretation for three specific animal tumor responses. These science policies addressed proliferative lesions in the rat liver (Rinde *et al.* 1987), male rat-limited kidney tumors associated with accumulation of alpha $_{2u}$-globulin (USEPA, 1991), and thyroid tumors resulting from disruption of thyroid-pituitary homeostasis (USEPA, 1997). Accumulated information on rodent liver tumors that are induced through the activation of the peroxisome proliferator-activated receptor α (PPARα) led the International Life Sciences Institute Risk Science Institute (ILSI RSI) to establish an expert working group to update the state of the science on PPARα agonist-induced carcinogenesis in rodents and the human relevance of such animal tumors (ILSI, in press). The report of this ILSI RSI workgroup provides the current scientific understanding of the mode(s) of action of three of the PPARα agonist-induced tumors observed in rodent bioassays; liver tumors in rats and mice and Leydig cell and pancreatic acinar cell tumors in rats.

There are a number of pesticides and industrial chemicals that increase peroxisomes (number and size) and liver tumors in rodents via activation of PPARα. This proposed guidance document is intended to provide direction to the scientists in the Office of Pollution, Prevention and Toxic Substances in evaluating liver tumor data observed following exposure to this subgroup of carcinogens. This document also responds to the EPA policy concerning risks to infants and children (USEPA, 1995; USEPA, 2003) which requires that each risk assessment present findings explicit for these lifestages. OPPTS will depart from the science policy within this document where the facts or circumstances warrant. In such cases, OPPTS will explain why a different course was taken.

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I. Introduction

There has been substantial scientific interest regarding the role of peroxisome proliferation in rodent hepatocarcinogenesis and its relevance for human carcinogenesis at this and other potential sites. Several scientific groups have examined the state of the science on PPARα agonist -induced rodent liver tumors over the years. A workgroup convened under the auspices of the International Agency for Research on Cancer concluded that the mode of action (MOA) for liver tumors induced in rodents by PPARα agonists is unlikely to be operative in humans (IARC, 1995). The participants of a workshop held under the auspices of the International Life Sciences Institute Health and Environmental Sciences Institute concluded that although it appeared unlikely that PPARα agonists could induce liver tumorigenesis in humans, the possibility could not be ruled out (Cattley *et al.* 1998).

Recent scientific developments have led to a reevaluation of the state of the science to characterize the mode(s) of action (*e.g.,* PPARα-agonism) and human relevance of rodent tumors induced by certain peroxisome proliferating agents. To that end, the ILSI Risk Science Institute convened a workgroup to upgrade the state of the science for PPARα agonist-induced rodent liver tumors, as well as to evaluate the mode(s) of action for Leydig cell and pancreatic acinar cell tumors, which also are observed frequently in rats with PPARα agonists. The workgroup provided a detailed analysis of the key events in the mode of action of PPARα-agonist induced liver tumors in rodents, and then proceeded with a concordance analysis of the evidence that these key events can occur in humans. The workgroup concluded that while the PPARα receptor can be activated in humans, there are substantial species differences in toxicodynamics that make it very unlikely that the downstream key events and therefore hepatocarcinogenesis would occur in humans. Finally, the workgroup concluded that there is insufficient information at this time to firmly establish a mode of action for the Leydig cell and pancreatic acinar cell tumors in rats (ILSI, in press). It should be noted that the ILSI expert panel's report on "PPARα Agonist-Induced Rodent Tumors: Mode(s) of Action and Human Relevance" was peer reviewed by an independent group of 17 scientists from academia, government and industry.

The purpose of this OPPTS guidance document is to describe the approach the Office will proposes to use to evaluate the scientific information regarding the mode of action of PPARα agonists in rodent hepatocarcinogenesis and the relevance of this mode of action for human hepatocarcinogenesis. Other tumor types (e.g. Leydig cell and pancreatic acinar cell tumors) that may be associated with PPARα-agonists are briefly described. The document provides an overview of the evidence for a PPARα-agonist mode of action for liver tumors in rodents, and an overview of all known age and species differences in the key events. Finally, the document will provide guidance in: 1) using a framework to describe and to present the proposed PPARα-mediated mode of action; 2) data needed to demonstrate that rodent liver tumors have arisen as a result of a PPARα agonist mode of action; and 3) the relevance of this mode of action for hepatocarcinogenesis in humans. OPPTS will depart from this proposed science policy where the facts and circumstances warrant.

II. Overview of Peroxisome Proliferation and PPARα Agonism

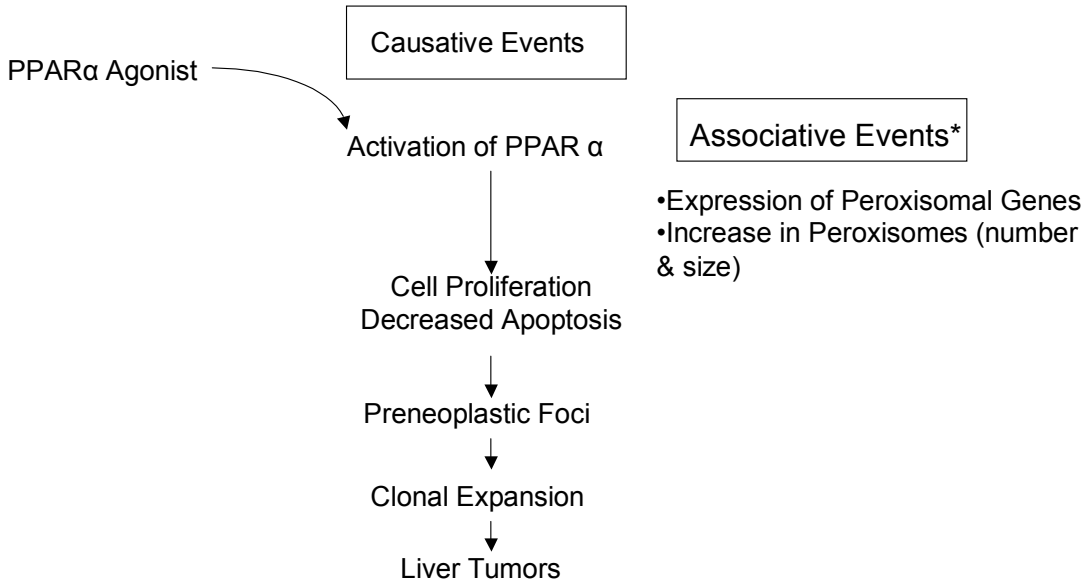
Events leading to carcinogenic response following exposure to an environmental agent are varied and may range from mutations of the genome to the activation/deactivation of genes and/or receptors. An example of this latter category is PPARα agonism. This event is key in rodent liver carcinogenesis triggered by administration of certain peroxisome proliferating agents. An increase in the number and volume of peroxisomes present in liver cells is a key characteristic of PPARα agonism. It is generally accepted that peroxisome proliferation is associated with liver tumor formation in rodents although this phenomenon has not been established as a causal effect.

In addition to inducing hepatocarcinogenesis in rodents, PPARα agonists have also been observed to induce pancreatic acinar cell and Leydig cell tumors in rats. Of 15 PPARα agonists tested to date, nine have been shown to induce all three tumors in non-F344 rat strains but **not** in mice. In the case of Leydig cell tumor formation, two potential MOAs based on activation of PPARα have been proposed. One MOA invokes the induction of hepatic aromatase activity leading to an increase in serum estradiol level. The second MOA purports that PPARα agonists inhibit testosterone biosynthesis. Although agonism of PPARα may lead to the induction of aromatase or inhibition of testosterone biosynthesis, the data available to date are insufficient to support which, if either, of these two proposed MOAs is operative. For pancreatic acinar cell tumor (PACT) formation, a MOA has been proposed in which PPARα-agonists cause a decrease in bile acid synthesis and/or change the composition of the bile acid resulting in cholestasis. These steps increase the level of the growth factor cholecystokinin (CCK) which then binds to its receptor, CCK_A, leading to acinar cell proliferation. Some evidence exists to support this proposed MOA and there does not appear to be evidence of any other MOA operating in the formation of PACTs after exposure to PPARα agonists. However, the data are not considered sufficient to establish a MOA with confidence, because it has only been described for two chemicals, PFOA and WY14643, in one laboratory. As a result, the evidence is considered insufficient to infer that this MOA may be generalized to all PACT-inducing PPARα agonists.

III. Establishing PPARα-Agonism as a Mode of Action for Rodent Hepatocarcinogenicity

Although the precise mechanistic steps leading to hepatic carcinogenesis in rodents after PPARα agonist exposure are not completely understood, knowledge of the mode of action has been characterized (ILSI, in press). As shown in Figure 1, it has been proposed that PPARα agonists activate PPARα (which regulates the transcription of genes involved in peroxisome proliferation, cell cycle control, apoptosis, and lipid metabolism). Activation of PPARα leads to an increase in cell proliferation and a decrease in apoptosis, which in turn leads to preneoplastic cells and further clonal expansion and formation of liver tumors. As depicted in Figure 1, the biological events in PPARα-induced hepatocarcinogenesis may be classified as either causal (*i.e.*, required for this MOA) or associative (*i.e.,* markers of PPARα agonism but not shown to be directly involved in the etiology of liver tumors). PPARα activation, changes in rates of liver cell proliferation/apoptosis, and selective clonal expansion are causally related to PPARα-mediated liver tumor formation. Of these, only PPARα activation is highly specific for this MOA while cell proliferation/apoptosis and clonal expansion are common to other modes of action. Among the associative events are peroxisome proliferation (a highly specific indicator that this MOA is operative) and peroxisomal gene expression. Peroxisomal proliferation may also result in hepatocyte oxidative stress which may contribute to the mode of action by causing indirect DNA damage and leading to mutations, or by stimulating cell proliferation. However, increases in oxidative damage to DNA have not been unambiguously demonstrated for PPARα agonists. Oxidative stress is a general phenomenom, and thus does not represent a highly specific marker for PPARα-agonist induced liver carcinogenesis. For a more detailed description of the PPARα agonist mode of carcinogenic action see ILSI (in press).

Figure 1. Key Events in the Mode of Action for PPARα Agonist Induced Rodent Liver Tumors



*Although there are other biological events (e.g., Kupffer cell mediated events, inhibition of gap junctions), the measurements of peroxisome proliferation and peroxisomal enzyme activity (in particular acyl-CoA) are widely used as reliable markers of PPARα activation.

When postulating a PPARα-agonist mediated MOA for rodent liver tumorigenesis, one of the first indications that this MOA may be operative is the increase in the number and size of peroxisomes in the cytoplasm of hepatocytes. It should be noted that although peroxisome proliferation itself has not been shown to be a causal event in liver tumorigenesis , it is a highly specific biomarker of the PPARα agonist MOA. Evidence of peroxisome proliferation (and by extension PPARα agonism) has also been obtained through biochemical assays - conducted as part of long term cancer studies or as independent mechanistic studies - that evaluate the activity of peroxisome-specific enzymes involved in the metabolism of fatty acids. These assays include activity level measurements for acyl-CoA oxidase, palmitoyl CoA oxidase, carnitine acetyl transferase, and CYP4A. Increases in palmitoyl CoA, cartinine acetyl transferase, and CYP4A activity have been noted in conjunction with liver tumorigenesis after exposure to certain peroxisome proliferators (PP) such as clofibrate, lactofen, trichloroacetic acid, oxidiazon, and di-(2-ethylhexyl)phthalate (DEHP)(ILSI, in press). Furthermore, the observation that acyl CoA oxidase induction after PPARα agonist exposure is not seen in PPARα-null mice, has served to identify changes in this enzyme activity as a key event in the induction of rodent liver tumors by PPARα-agonists and a biomarker for the MOA (Ward *et al.* 1998). In addition to biochemical alterations, other liver changes noted after exposure to PPARα agonists include: hepatocyte hypertrophy, increased liver weights, and increased liver cell mitotic activity (reviewed in Cattley *et al.* 1998).

The discovery and cloning of the PPARα in 1990 by Isseman and Green provided insight into the role of PPARα in the processes leading to hepatocarcinogenesis in rodents (Isseman and Green, 1990). Conclusive evidence of the pivotal/causal role of PPARα in the induction of liver tumors by certain PPARα agonists was obtained from studies on PPARα-null knockout mice. In a series of seminal experiments, Lee *et al.* (1995) and Peters *et al.* (1997) exposed wild type (+/+) and PPARα-null (-/-) mice to either WY14643 or clofibrate - two known PPARα agonists - at doses known to induce hepatocarcinogenesis in rodents. Short-term exposure (2 weeks) to either one of these PPARα agonists failed to elicit in PPARα-null mice the early hepatic effects characteristic of liver tumor induction such as increases in liver weights, hepatic peroxisome proliferation, CYP4A induction as well as induction of other peroxisomal enzymes. In contrast, wild type (+/+) mice displayed all these indicators of hepatocellular alteration. These findings were corroborated by experiments using longer periods of exposure (5 weeks to 11 months) in which WY14643 exposure failed to elicit in PPARα-null mice the increases in acyl CoA oxidase, CDK-1, CDK-4, and *c-myc* seen in wild type mice. WY14643 treatment also failed to induce replicative DNA synthesis (as measured through BrDU labeling indices) in PPARα-null mice under conditions that elicited this effect in the wild type mice. Moreover, WY14643 exposure for 11 months led to a 100% incidence of hepatic neoplasms in wild type mice while the PPARα-null mice showed none. PPARα-null mice exposed to DEHP for 24 weeks did not manifest any of the changes associated with PPARα-mediated liver tumors such as peroxisome proliferation, increased peroxisomal enzyme activity, or increased CYP4A mRNA levels (Ward *et al.* 1998). Experiments on null mice after WY14643, clofibrate, or DEHP exposure provide persuasive evidence that a PPARα-mediated MOA is operating in the induction of hepatocellular neoplasms.

IV. Human and Non-human Primate Response to PPARα Agonists

Studies conducted in numerous test species indicate that while rodents (mice and rats) are highly responsive to PPARα agonist-induced hepatocarcinogenicity, other species (*e.g.,* hamster, dogs, guinea pigs, New and Old World primates, and humans) appear to be refractory (Cattley *et al.* 1998; Doull *et al.* 1999). For example, a wide range of PPARα agonists which produce peroxisome proliferation in rodent hepatocytes, have little or no effect in guinea pig or monkeys when the test compounds were evaluated by changes in peroxisome proliferation and/or enzyme activities (*e.g.,* palmitoyl-CoA) (Doull *et al.* 1999). Furthermore, liver tumors are not found in Syrian hamsters after 40-60 weeks administration of WY14643 and nafenopin (Lake *et al.* 1993). A variety of studies have been conducted to characterize the response of humans and non-human primates to PPARα agonists, and to determine the cause(s) of the apparent refractory nature of the response. These studies are summarized below.

Two major epidemiology studies have been conducted to assess the effect of prolonged exposure to the hypolipidemic drugs gemfibrozil or clofibrate. In the Helsinki Heart Study over 4000 men with high cholesterol were treated with gemfibrozil or a placebo for five years. Although substantial decreases in the serum lipid levels were noted in the gemfibrozil treated group compared to the placebo group, the death rate and liver cancer rates in both groups were comparable. It is noteworthy, however, that

in this study the liver cancer incidence was reported in conjunction with gall bladder and intestinal cancers (Frick *et al.*, 1987; Huttunen *et al.*, 1994).

In the second study, over 15,000 men with ischemic heart disease received clofibrate or a placebo for five years, and their health status was followed for eight years after cessation of treatment (CPI,1984). During the follow up conducted 4 years after treatment stopped, the group treated with clofibrate had a 24% increase in mortality relative to the high cholesterol patients treated with placebo. These mortalities were attributed to diseases (including malignancies) of the gall bladder, intestines, liver and, pancreas. Eight years after treatment was stopped, however, the cancer death rate between the two groups was comparable. As was the case in the Helsinki Heart Study, cancer data were not provided specifically for the liver but rather they were presented in combination with the gall bladder, pancreatic, and intestinal cancer incidences. Thus, neither of these epidemiological studies has provided evidence of PPARα-mediated hepatocarcinogenesis; however, these data are inconclusive because of the limited durations of exposure

The apparent species differences between rodents and human and nonhuman primates in the response to PPARα agonists is also supported by a long-term study in monkeys. Marmosets exposed for 6.5 years to clofibrate at relatively high doses (94 mg/kg/day or higher) did not develop liver tumors (Tucker and Orton, 1995). Although the duration of this study did not represent a lifetime exposure, the results strongly suggest that primates appear to be refractory to PPARα agonists (Doull *et al.*, 1999 and ILSI, in press).

Examination of liver biopsies from patients receiving hypolipidemic drugs (clofibrate or ciprofibrate) to treat hyperlipidemia provide another line of evidence for human response to PPARα agonists. Clofibrate exposure - at clinically relevant doses - has been shown only to induce a 1.5-fold increase in the number of hepatic peroxisomes and a 23% increase in the volume density of these organelles. Similar findings have been reported for ciprofibrate where liver biopsies from patients treated with the drug for 6 months to two years exhibited a 30% increase in the volume density of hepatic peroxisomes (Bentley *et al.*, 1993; Hinton *et al.*, 1983). In addition, there was no evidence of liver tumors from liver biopsies that were conducted on patients treated with gemfibrozil, clofibrate or fenofibrate for periods ranging for up to 5.3 years and followed up for an additional 7.9 years (Blumcke *et al.*, 1983; De La Iglesia *et al.*, 1982; Gariot *et al.*, 1987).

Further evidence that humans appear to be refractory to the hepatocarcinogenic properties of PPARα agonists is provided by experiments in which exposure to ciprofibrate, clofibrate, gemfibrozil, and fenofibrate (hypolipidemic drugs known to be PPARα agonists) failed to elicit a response in human hepatocytes that is observed in rodent hepatocytes. Human hepatocytes cultured in the presence of these compounds did not exhibit increases in replicative DNA synthesis or acyl CoA oxidase activity and/or expression (*i.e.* mRNA levels), suppression of apoptosis, or increased peroxisome proliferation. These findings are consistent with the results obtained after exposure to other PPARα agonists that are not hypolipidemic drugs such as mono-2-ethylhexyl

phthalate (MEHP) (Baker *et al.*, 1996; Kamendulis *et al.*, 2002; Hasmall et al. 1999 and 2000).

In vitro and *in vivo* data on monkeys (cynomolgus, marmoset, and Rhesus) support the findings from human *in vitro* and liver biopsy studies. Palmitoyl CoA oxidase activity was evaluated in monkeys after *in vivo* exposure to a variety of PPARα agonists (e.g., bezafibrate, clofibrate, di-2-ethylhexyl phthalate (DEHP), MEHP, fenofibrate, nafenopin, and LY171883). The changes noted in enzymatic activity after PPARα agonist exposure were minimal or non-existent relative to control (ILSI, in press). Moreover, cynomolgus monkeys exposed *in vivo* to DEHP, di-isononyl phthalate and clofibrate also failed to exhibit an increase in DNA synthesis (Pugh *et al.*, 2000). It should be noted, however, that cynomolgus monkeys treated for two weeks with clinically relevant doses of fenofibrate or ciprofibrate exhibited a 3-fold increase in the number of hepatic peroxisomes, a 2-fold increase in liver size, and hepatocellular hypertrophy (Qualls *et al.*, 2003). Although these changes appear to be similar to the response in rodents to PPARα agonist exposure, there was no evidence of increases in peroxisomal enzyme activity (*e.g.,* acyl CoA oxidase, catalase, or carnitine acetyltransferase).

Research on several aspects of PPARα agonism has been undertaken to attempt to ascertain the basis for the differential response between rodents and humans after exposure to PPARα agonists. These investigations have focused on PPARα gene expression, PPARα gene structure, and peroxisome proliferator response element (PPRE) structure. After the discovery and cloning of the gene encoding the human PPARα, Tugwood *et al.* (1996) and Palmer *et al.* (1998) demonstrated that PPARα expression in humans is 10-fold lower than that seen in rodents. While this may account in part for the unresponsiveness of humans to the hepatocarcinogenic properties of PPARα agonists, it is unknown how much it contributes to the differences between the species.

Transfection experiments have demonstrated that human PPARα (hPPARα) is very similar to the rodent PPARα and capable of transactivating reporter genes (containing a rodent PPRE in their promoter region) following clofibrate exposure, thereby establishing that hPPAR" is functional (Sher *et al.*, 1993; Mukherjee *et al.*, 1994; Pineau *et al.*, 1996). This finding was confirmed by *in vivo* experiments in which PPARα-null mice were infected by an adenovirus carrying the hPPARα gene. The hPPARα gene was able to “rescue” these knockout mice as evidenced by the increases in peroxisome proliferation and peroxisomal enzyme gene expression (Yu *et al.*, 2001).

Other studies have focused on the potential variability in the structure of the PPARα gene in human populations. The hPPARα 8/14 variant identified by Tugwood *et al.* (1996) results in a truncated hPPARα, while hPPARα 6/29, identified by Roberts *et al.* (1998), results in a four amino acid substitution. The hPPARα 8/14 variant has been detected in every sample examined to date in two laboratories while hPPARα 6/29 is extremely rare having been isolated only once (Roberts *et al.*, 2000). Two point mutations (PPARα*3 and PPARα*2) were identified by Sapone *et al.* (2000). PPARα*3 is relatively common in Northern India populations while PPARα*2 is relatively rare.

Relative to wild type, PPARα*3 results in unresponsiveness to low concentrations of ligand as well as a lower non-ligand dependent trans-activating (*i.e.* constitutive) activity. In contrast, PPARα*2 exhibits a slightly higher constitutive activity than wild type. However, PPARα*2 activity after WY14643 exposure is comparable to wild type. Precisely how these mutations may render humans less sensitive to PPARα agonists is not known.

Another factor that may contribute to the apparent lack of response in humans to the hepatocarcinogenic inducing properties of PPARα agonists may be related to a difference in the hPPREs response to hPPARα induction. This theory stems from transfection studies in which Sher *et al.* (1993) demonstrated that hPPARα can transactivate reporter genes containing a murine PPRE. However, the gene and protein expression of various biomarkers indicative of PPARα-mediated liver tumorigenesis and regulated by hPPRE, including acyl CoA oxidase, were not increased in human hepatocytes following exposure to several PPARα agonists. These findings were corroborated by data collected after patients were exposed at clinically relevant doses to various fibrates (*e.g.,* bezafibrate, fenofibrate, and gemfibrozil) which indicate that acyl CoA oxidase mRNA levels were unaffected (Roglans *et al.,* 2002). In contrast, other human genes regulated by PPARα agonists - but not implicated in hepatic carcinogenesis - (*e.g. ,* lipoprotein kinase, carnitine palmitoyl transferase, etc.) are effectively regulated by PPARα agonists (discussed in ILSI, in press). Moreover, in experiments in which HepG2 cells were transfected with hPPARα (at the levels of PPARα seen in rodents) and treated with fibrate did not lead to increases in acyl CoA oxidase mRNA levels (Hsu *et al.*, 2001; Lawrence *et al.*, 2001a). The possibility that the hPPRE for acyl CoA oxidase may differ from the rodent in its ability to bind hPPARα has been further suggested by experiments showing that this peroxisomal enzyme cannot be activated in cells transfected with a PPARα expression vector and that it may require high concentrations of PPARα agonists to be induced (Woodyatt *et al.*, 1999; Varanasi *et al.*, 1998; Rodriguez *et al.*, 2000). Furthermore, Lambe *et al.* (1999) have shown that when rat acyl CoA oxidase PPRE is disrupted by site directed mutagenesis, the hPPRE for this enzyme could not rescue the null phenotype in reporter gene assays. Although it appears that the downstream effects of PPARα agonism such as induction of acyl CoA oxidase, CYP4A1, DNA replication, or apoptosis suppression do not occur in humans, hPPARα in COS-1 or NIH 3T3 cells can be activated by exposure to MEHP as indicated by the increases in luciferase reporter gene activity (Dirven *et al.*, 1993; Maloney & Waxman, 1999; Hasmall *et al.*, 2000).

In summary, although humans possess a functional PPARα, and the human receptor can be activated by peroxisome proliferators, humans (and non-human primates) appear to be refractory to the key events associated with the induction of liver tumors by PPARα agonists. It is the totality of evidence that provides a strong argument that PPARα-induced liver carcinogenesis is not likely to occur in humans. Human and non-human primate hepatocytes treated with a range of different PPARα agonists do not exhibit increased replicative DNA synthesis, suppression of apoptosis, increased expression of marker mRNAs and proteins including acyl CoA oxidase or peroxisome proliferation. *In vivo* studies with monkeys showed that treatment with the hypolipdemic drugs fenofibrate or ciprofibrate induced an increase in liver weights (up to

2-fold) and peroxisome numbers (up to about 3-fold), but only slight or no increases in peroxisomal enzyme activities. Similarly, analyses of biopsies from liver tissue of patients treated with hypolipodemic drugs showed only slight increases or no increases in peroxisome numbers or volume density. Although the epidemiological literature and cancer studies in nonhuman primates are inconclusive, they do not provide evidence for the potential of PPARα agonists to induce liver tumors in humans.

V. Development of PPARα Activity and Responses to PPARα Agonists in the Fetus and Neonate

Previous sections of this document provided an overview of the evidence and basis for the establishment of PPARα-agonism as a mode of action for the induction of liver tumors observed in adult rodents. This section focuses on the ontogeny of the response to PPARα agonists during fetal and postnatal development and the sensitivity of the developing organism relative to the adult. No data on the ontogeny of the response during human development were found in the literature. Thus, conclusions concerning the ontogeny of the response to PPARα agonists and the relative sensitivity of developing humans must rely on evidence provided by laboratory studies in animals.

Ontogeny of PPAR α Expression, Peroxisomal Assemblage, Peroxisomal Numerical or Volume Density, and Peroxisomal Enzyme Activities

A detailed review of studies available on the ontogeny of peroxisomes and peroxisomal enzyme activities during development in the rodent liver is provided in Appendices A-1 and A-2. These studies indicate that expression of the PPARα gene, assemblage of peroxisomal proteins into plasmids, the content of peroxisomes, and peroxisomal enzyme activity appears to occur late in rodent fetal development (i.e., gestational day 15 or later) (Braissant and Wahli, 1998; Wilson et al, 1991; Tsukada, *et al.*, 1968; Stefanini *et al.*, 1989; Stefanini, *et al.*, 1985; Cibelli, *et al.*, 1988). Thus, it appears that the direct effects of a PPARα agonist on PPARα and secondary effects on peroxisomal proliferation and enzyme activites would not be operative prior to gestational day 15 or later.

In the neonatal rat, peroxisomal assemblage, peroxisomal numerical density and the volume of peroxisomes have been reported to be similar among neonatal and adult rats, as have peroxisomal enzyme activities (Stefanini *et al.*, 1999; Stefanini *et al.*, 1995; Cimini *et al.*, 1994; Singh and Lazo, 1992; Dostal *et al.*, 1987; Staubli *et al.*, 1977; Weibel *et al.*, 1969).

Based on the available evidence, it appears that PPARα expression is low or absent in the rodent fetus until just before birth. In neonatal rodents, the expression of PPARα mRNA is comparable to or less than that seen in the adult rodent. Thus, it is plausible that rodent embryos would not respond to the effects of a PPARα agonist until late in development whereas the neonate might be expected to respond to a PPARα agonist like adult rodents.

PPARα Agonism in the Fetus and Neonate

Several studies have been conducted to examine the effects of exposure to PPARα agonists during fetal and postnatal development in rats and mice. These studies are reviewed in detail in Appendices A-3 - A-5. Administration of the PPARα agonist clofibrate to rats or mice during pregnancy can accentuate peroxisome assemblage or peroxisomal proliferation in the liver of their fetuses late in development (i.e., during gestational days 17-21)(Wilson *et al.*, 1991; Stefanini *et al.*, 1989; Cibelli, *et al.*, 1988). Markers of peroxisomal assemblage, the proteins PMP-70 and DHAP-AT, are increased, relative to controls, to an equal or greater extent in the mouse fetus compared to clofibrate-treated dams. However, the total levels of these assemblage proteins (*i.e.,* on a µg/mg or units/mg protein basis) are lower in the fetus than in treated dams. Peroxisomal numbers also increased in rat or mouse fetuses from dams treated with clofibrate, particularly in gestational day 19 fetuses. The activity of catalase, a peroxisomal enzyme which is also found in the cytoplasm, increases somewhat in gestational day 19 to 21 fetuses and neonates from mothers treated with clofibrate but the specific activity of the enzyme does not exceed the level of activity observed in clofibrate-treated rat or mouse dams. Similarly, palmitoyl CoA oxidase activity was enhanced as much as 8-fold in gestational day 21 rat fetuses but, again, the enhanced specific activity of the enzyme was about equal to that seen in the clofibrate-treated dams.

Although limited, these data when considered together suggest that: 1) the rodent fetus responds to a PPARα agonist like the adult rodent dam (*i.e*., there are increases in peroxisomal assemblage/proliferation and peroxisomal enzyme activities; 2) effects of a PPARα agonist on the fetus occur late in development; and 3) although the enhancement, relative to controls, of peroxisomal numbers and peroxisomal enzyme activities during fetal development may exceed that observed in the PPARα agonist-treated pregnant rat or mouse, the increased levels of peroxisomal protein or peroxisomal enzyme activities do not exceed the levels found in the pregnant dam. Thus, it does not appear that the fetal rodent is more sensitive than the adult rodent dam to the effects of a PPARα agonist.

Treatment of rat dams during lactation also leads to an enhancement of peroxisomal assemblage, peroxisomal enzyme activities, and the numerical density of peroxisomes in their nursing pups (Stefanini *et al.*, 1999; Stefanini *et al.*, 1995; Cimini *et al.*, 1994; Singh and Lazo, 1992; Fahl *et al.*, 1983). Generally, nursing neonates appear no more sensitive than their PPARα agonist treated dams because increases in these parameters are similar to the increases observed in the treated dams. Furthermore, increases in liver weights, an effect characteristic of PPARα agonism, in nursing neonates exposed to a PPARα agonist were no greater or less than that reported in treated dams (Singh and Lazo, 1992; Cimini *et al.*, 1994; Osterburg *et al.*, 1992; Schroeder, 1983). At this time, however, it is unknown how much of the compound is metabolized by the dams and/or transferred to the neonates via the milk; hence it is possible that a difference in the internal dose may play a role in the differential responses noted between nursing neonates and their dams.

Direct exposure of neonates to PPARα agonists results in an increase in peroxisomal enzyme activities and an increase in the numerical density or volume of

peroxisomes; the increases in these parameters are comparable to those observed in young adults or adult rats (Yu *et al.*, 2001;Yamoto, 1996; Dostal *et al.*, 1987; Staubli *et al.,* 1977;). At a dose of a PPARα agonist that affects peroxisome enzyme activity or peroxisome numbers, no effect on liver weights was observed (Yu *et al.*, 2001) or the increase in liver weights observed in neonates was no greater than that observed in treated adults (Dostal *et al.*, 1997; Yamoto, 1996)

Conclusions

The data available on the effects of PPARα agonist in the rodent fetus or neonate (e.g., increases in peroxisome numbers and size, peroxisome enzyme activities, and liver weights) provides support that there is not an increased sensitivity relative to an adult to hepatocarcinogenicity during fetal or neonatal development. Any conclusions regarding this hepatocarcinogenic mode of action in adult rodents would also appear to apply to young rodents, and similarly any conclusions regarding the relevance of this mode of action for human hepatocarcinogenesis would apply to the young, as well as the adults.

VI. Proposed Science Guidance

Proposed Science Policy Statements

Although the precise mechanism for the formation of liver tumors by a PPARα agonist has not been established, key events for the mode of action leading to hepatocarcinogenesis have been identified. Key events for the mode of action that have been causally related to liver tumor formation include: activation of PPARα, perturbation of cell proliferation and apoptosis, and selective clonal expansion. Key events that are associated with PPARα agonism and liver tumor formation and that are reliable markers that a chemical has induced PPARα include expression of peroxisomal genes (*e.g.,* palmitoyl CoA oxidase, acyl CoA oxidase) and peroxisome proliferation (*i.e.,* an increase in the number and size of peroxisomes).

It has been well established that chemicals that are PPARα agonists can induce liver tumors, and perhaps other tumors, in rats and mice but the potential for PPARα agonists to induce liver tumors in other species, including humans, appears to be unlikely. This is because evidence obtained from *in vivo* and *in vitro* studies with hamsters, guinea pigs, non-human primates, and humans (*i.e.*, cells in culture or biopsies) shows that, quantitatively, these other species are apparently refractory to the effects of a PPARα agonist. Lines of evidence supporting this presumption include minimal or no effects on peroxisome proliferation, peroxisomal enzyme activity, or hepatocellular proliferation in species other than rats or mice. Moreover, epidemiological studies have not provided evidence of increased incidence of liver neoplasms in humans; however, these data are inconclusive because of the limited durations of exposure.

Recognizing the mode of action data that show a linkage between PPARα agonism and liver tumor formation, OPPTS proposes to adopt the following science policy:

- When liver tumors are observed in long term studies in rats and mice, and 1) the data are sufficient to establish that the liver tumors are a result of a PPARα agonist MOA and 2) other potential MOAs have been evaluated and found not operative, the evidence of liver tumor formation in rodents should not be used to characterize potential human hazard.

- There is limited evidence that a chemical may induce pancreatic and Leydig cell tumors through a PPARα agonist mode of action. However, the evidence is inadequate at this time to support a linkage between PPARα agonism and formation of these tumor types. Thus, it is presumed that chemicals in this subclass that induce pancreatic or Leydig cell tumors may pose a carcinogenic hazard for humans.

Different types of data on a chemical may be provided that indicate that a chemical induces liver tumors via a PPARα agonist mode of action. The approach to establishing that a PPARα agonist mode of action is operative and the data needed to support this presumption are discussed below.

Framework to Establish the PPARα Agonist MOA

The Agency uses an analytical framework for judging whether available evidence for an agent supports a mode of action for tumor induction in animals (USEPA, 1999, Sonich-Mullin *et al.*, 2000). This framework was considered in evaluating the postulated mode of action for PPARα agonists.

The framework for analyzing mode of action begins with a summary description of the postulated mode(s) of action. This is followed by questions to be addressed to the available empirical data and experimental observations anticipated to be pertinent. The areas of inquiry in the framework are:

(i) identification of key event(s). A “key event” is defined as an empirically observable, precursor step that is a necessary element of the mode of action, or is a marker for such an element (*e.g.*, increased cell growth and organ weight, hyperplasia, cellular proliferation, hormone or other protein perturbations, receptor-ligand changes, DNA or chromosome effects, cell cycle effects).,

(ii) strength, consistency, specificity of association (*e.g.,* causality is supported by a significant statistical and biological association between key events and a tumor response in well conducted studies and with consistent observations in a number of such studies, with differing experimental designs),

(iii) dose-response relationships (i.e., key event(s) and tumor response increase correlatively with dose),

(iv) temporal relationships (i.e., if an event(s) is an essential element of tumorigenesis, it should precede tumor appearance),

(v) biological plausibility and coherence (i.e., is the mode of action consistent with what is known about carcinogenesis in general and for the case specifically?),

(vi) other modes of action (i.e., have alternative modes of action for the tumor response been considered and are they supported by the data?).

It should be emphasized that the topics described above for analysis should **not** be regarded as a checklist of necessary "proofs". The judgment whether a postulated mode of action is supported by available data takes into account the weight of the evidence and the analysis as a whole.

Data Needs

Chemicals can produce tumors at a given site by more than one mode of action. Thus, before a PPARα agonist MOA can be defined as a cause of the liver tumors, it is also critical to ensure that other MOAs do not contribute significantly to the development of the tumors. For instance, it is important to ensure that direct DNA reactivity is not the source of the carcinogenic findings. The results of *in vitro* and *in vivo* short term tests for mutagenicity and the evaluation of the presence of structural alerts and structure-activity relationships are helpful. Likewise, chemicals producing rodents liver tumors exclusively through PPARα activation do not cause cytotoxicity; such findings need to be evaluated at doses that have produced PPARα agonist precursor effects and liver tumors to ensure that cytotoxicity is not prominent.

Parameters chosen to demonstrate that activation of PPARα is the mode of action for the induction of rodent liver tumors must be both sensitive and specific. In other words, precursor events, whether causally related to or associated with liver tumor formation, must clearly show that tumors are due to a PPARα agonist MOA and exclude other potential MOAs. Valuable data to address specificity can also be obtained from PPARα knockout mouse bioassays. Demonstration of the absence of hepatocarcinogenicity and related liver toxicity in a PPARα knockout mouse but the presence of liver tumors and toxic responses in the wild type mouse not only provides evidence that a PPARα agonist MOA is operating in the induction of the liver tumors but also demonstrates that other MOAs (*e.g.,* direct mutagenic effects, cytotoxicity) are not major contributors to the onset of hepatocarcinogenesis. However, it is recognized that a PPARα knockout mouse is not generally used to demonstrate that a chemical induces liver tumors via a PPAR α agonist MOA. Thus, other data may be used to demonstrate that a PPAR α agonist MOA for liver tumor formation is operative.

Demonstration that a PPARα agonist MOA is operative can be shown by a data set that includes *in vitro* evidence of PPARα agonism (*i.e.,* evidence from an *in vitro* receptor assay), *in vivo* evidence of an increase in number and size of peroxisomes, increases in the activity of acyl CoA oxidase, and hepatic cell proliferation. The *in vivo* evidence should be collected from studies designed to provide the data needed to show

dose-response and temporal concordance between precursor events and liver tumor formation.

Because some chemicals have been shown to induce little or no effect on PPARα activity but produce other effects associated with PPARα agonisms (ILSI, in press), a receptor assay should be used to demonstrate that a compound is a PPARα agonist and is not inducing effects via other PPAR receptors. Evidence of peroxisome proliferation is a fundamental aspect of PPARα agonists and along with evidence from an in vitro reporter assay provides definitive evidence that a PPARα agonist MOA is operative. Increases in peroxisomal enzyme activities are commonly used markers for peroxisome proliferation and data from measurements of peroxisomal enzyme activity, when combined with direct evidence of peroxisome proliferation, enhance the ability to establish temporal and dose-response concordance between key events and liver tumor formation. Finally, hepatic cell proliferation is a key, causal event leading to the formation of liver tumors by PPARα agonists and evidence of hepatic cell proliferation, when combined with other evidence, may also provide important information on the temporal aspect of tumor development and the dose-response concordance of precursor events and tumor formation.

Other information that is desirable and may strengthen the weight of evidence for demonstrating that a PPARα agonist MOA is operative includes data on hepatic CYP4A1 induction, palmitoyl CoA activity, hepatocyte hypertrophy, increase in liver weights, decrease in the incidence of apoptosis, increase in microsomal fatty acid oxidation, and enhanced formation of hydrogen peroxide.

REFERENCES

Baker, T., Kalimi, G., Lington, A., Isenberg, J., Klaunig, J., Nikiforov, A. (1996). Gap junctional intercellular communication (GJIC) studies on 5 phthalate monoesters in hepatocytes of four species: Implications for cancer risk assessment. *The Toxicologist*, An Official Publication of the Society of Toxicology and Abstract Issue of Fundamental and Applied Toxicology. **30**, 208.

Barber, E. D., Astill, B. D., Moran, E. J., Schneider, B. F., Gray, T. J. B., Lake, B. G., Evans, J. G. (1987). Peroxisome Induction Studies On Seven Phthalate Esters. *Toxicol. Ind. Health*. **3**, 7-21.

Blumcke, S., Schwartzkopff, W., Lobeck, H., Edmondson, N. A., Prentice, D. E., Blane, G. F. (1983). Influence of fenofibrate on cellular and subcellular liver structure in hyperlipidemic patients. *Atherosclerosis*. **46**, 105-116.

Braissant, O. and Wahli, W. (1998). Differential expression of peroxisome proliferator-activated receptor-alpha, -beta, -gamma during rat embryonic development. *Endrocrinology* **139(6):**2748-2754.

Cattley, R. C., DeLuca, J., Elcombe, C., Fenner-Crisp, P., Lake, B. G., Marsman, D. S., Pastoor, T. A., Popp, J. A., Robinson, D. E., Schwetz, B., Tugwood, J., Wahli, W. (1998). Do peroxisome proliferating compounds pose a hepatocarcinogenic risk to humans? *Regul. Toxicol. Pharmacol.* **27**, 47-60.

Cibelli, A., Stefanini, S., and Ceru, M.P. (1988). Peroxisomal β-oxidation and catalase activities in fetal rat liver: effect of maternal treatment with clofibrate. *Cell. Mol. Biol.* **34:**191-205.

Cimini, A.M., Sulli, A., Stefanini, S., Serafini, B., Moreno, S., Rossi, L., Giorgi, M., and Ceru, M.P. (1994). Effects of di-(2-ethyl-hexyl)phthalate on peroxisomes of liver, kidney, and brain of lactating rats and their pups. *Cell. Mol. Biol.* **40(8):**1063-1076.

CPI. Committee of Principal Investigators (1980). WHO cooperative trial on the primary prevention of ischemic heart disease using clofibrate to lower serum cholesterol: mortality follow-up. *Lancet*. **ii**, 379-385.

CPI. Committee of Principal Investigators (1984). WHO cooperative trial on primary prevention of ischaemic heart disease with clofibrate to lower serum cholesterol: final mortality follow-up. *Lancet*. **ii**, 600-604.

CPSC. Consumer Product Safety Commission (2001). Report to the U.S. Consumer Product Safety Commission by the Chronic Hazard Advisory Panel on Diisononyl Phthalate (DINP).

David, R. M., Moore, M. R., Cifone, M. A., Finney, D. C., Guest, D. (1999). Chronic peroxisome proliferation and hepatomegaly associated with the hepatocellular tumorigenesis of di(2-Ethylhexyl)phthalate and the effects of recovery. *Toxicol. Sci.* **50**, 195-205.

De La Iglesia, F. A., Lewis, J. E., Buchanan, R. A., Marcus, E. L., McMahon, G. (1982). Light and electron microscopy of liver in hyperlipoproteinemic patients under long-term gemfibrozil treatment. *Atherosclerosis*. **43**, 19-37.

Dirven HA, van den Broek PH, Peeters MC, Peters JG, Mennes WC, Blaauboer BJ, Noordhoek J, Jongeneelen FJ. (1993). Effects Of The peroxisome proliferator mono(2-ethylhexyl)phthalate in primary hepatocyte cultures derived from rat, guinea pig, rabbit And monkey. *Biochem*. *Pharmacol* **45**, 2425-2434,.

Doull, J., Cattley, R., Elcombe, C., Lake, B. G., Swenberg, J., Wilkinson, C., Williams, G., van Gemert, M. (1999). A cancer risk assessment of di(2-ethylhexyl)phthalate: application of the new U.S. EPA Risk Assessment Guidelines. *Regul. Toxicol. Pharmacol.* **29**, 327-357.

Dybing, E., Mikalsen, S.-O., Huttunen, J., Sanner, T. (1995). Peroxisome proliferation, genotoxicity and carcinogenicity. *IARC Technical Report.* **24**, 55-85.

Fahl, W.E., Lalwane, N.D., Reddy, M.K., and Reddy, J.K. (1983). Induction of peroxisomal enzymes in liver of neonatal rats exposed to lactating mothers treated with hypolipidemic drugs. *Biochem. J.* **210:**875-883.

Flavell, D.M., Pineda Torra, I., Jamshidi, Y., Evans, D., Diamond, J.R., Elkeles, R.S., Bujac, S.R., Miller, G., Talmud, P.J., Staels, B., Humphries, S.E. (2000). Variation in the PPARalpha gene is associated with altered function *in vitro* and plasma lipid concentrations in Type II diabetic subjects. *Diabetologia* **43(5):**673-680.

Frick, M. H., Elo, O., Haapa, K., Heinonen, O. P., Heinsalmi, P., Helo, P., Huttenen, J. K., Kaitaniemi, P., Koskinen, P., Manninen, V., Mäenpää, H., Mälkönen, M., Mäntttäri, M., Norola, S., Pasternack, A., Pikkareinen, J., Romo, M., Sjöblom, T., Nikkila, E. A. (1987). Helsinki Heart Study: primary prevention tool with gemfibrozil in middle-aged men with dyslipidemia. Safety of treatment, changes in risk factors, and incidence or coronary heart disease. *N. Engl. J. Med.* **317**, 1237-1245.

Gariot, P., Barrat, E., Drouin, P., Genton, P., Pointel, J. P., Foliguet, B., Kolopp, M., Debry, G. (1987). Morphometric study of human hepatic cell modifications induced by fenofibrate. *Metabolism*. **36**, 203-210.

Hasmall, S. C., James, N. H., Macdonald, N., Gonzalez, F. J., Peters, J. M., Roberts, R. A. (2000**a**). Suppression of mouse hepatocyte apoptosis by peroxisome proliferators: role of PPARα and TNF. *Mutat. Res.* **448**, 193-200.

Hasmall, S. C., James, N. H., Macdonald, N., Soames, A. R., Roberts, R. A. (2000**b**). Species differences in response to diethylhexylphthalate: suppression of apoptosis, induction of DNA synthesis and peroxisome proliferator activated receptor alpha-mediated gene expression *Arch. Toxicol.* **74**, 85-91.

Hasmall, S. C., James, N. H., Macdonald, N., West, D., Chevalier, S., Cosulich, S. C., Roberts, R. A. (1999). Suppression of apoptosis and induction of DNA synthesis *in vitro* by the phthalate plasticizers monoethylhexylphthalate (MEHP) and diisononylphthalate (DINP): a comparison of rat and human hepatocytes *in vitro*. *Arch. Toxicol.* **73**, 451-456.

Hinton, R. H., Mitchell, D. E., Mann, A., Chescoe, D., Price, S. C., Mann, A., Grasso, P., Bridges, J. W. (1986). Effect of phthalic acid esters on the liver and thyroid. *Environ. Health Perspect.* **70**, 195-210.

Hsu, M. H., Savas, U., Griffin, K. J., Johnson, E. F. (2001). Identification of peroxisome proliferator-responsive human genes by elevated expression of the peroxisome proliferator-activated receptor alpha in HepG2 cells. *J. Biol. Chem.* **276**, 27950-27958.

Huttunen, J. K., Heinonen, O. P., Manninen, V., Koskinen, P., Hakulinen, T., Teppo, L., Manttari, M., Frick, M. H. (1994). The Helsinki Heart Study: an 8.5-year safety and mortality follow-up [see comments]. *J. Intern. Med.* **235**, 31-39.

IARC (1995). *IARC Technical Report No. 24. Peroxisome Proliferation and its Role in Carcinogenesis*. WHO International Agency for Research on Cancer. IARC Press, Lyon, France.

ILSI (in press). PPARα agonist-induced rodent tumors: mode(s) of action and human relevance. ILSI RSI, Washington, DC

Issemann, I., and Green, S. (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*. **347**, 645-650.

Kamendulis, L., Isenberg, J., Smith, J., Pugh, G., Lington, A., Klaunig, J. (2002). Comparative effects of phthalate monoesters on gap junctional intercellular communication and peroxisome proliferation in rodent and primate hepatocytes. *J. Toxicol. Environ. Health A*. **65**, 569-588.

Kaufmann, W., Deckardt, K., McKee, R., Butala, J., Bahnemann, R. (2002) Tumor induction in mouse liver - Di-isononyl phthalate (DINP) acts via peroxisome proliferation. *Regul. Toxicol. Pharmacol.* 36: 175-183.

Lake, B. G., Evans, J. G., Cunninghame, M. E., Price, R. J. (1993). Comparison of the hepatic effects of nafenopin and WY 14,643-14,643 on peroxisome proliferation and cell replication in the rat and Syrian hamster. *Environ. Health Perspect.* **5**, 241-247.

Lambe, K. G., Woodyatt, N. J., Macdonald, N., Chevalier, S., Roberts, R. A. (1999). Species differences in sequence and activity of the peroxisome proliferator response element (PPRE) within the acyl CoA oxidase gene promoter. *Toxicol. Lett.* **110**, 119-127.

Lawrence, J., Li, Y., Chen, S., DeLuca, J. G., Berger, J. P., Umbenhauer, D. R., Moller, D. E., Zhou, G. (2001a). Differential gene regulation in human versus rodent hepatocytes by PPAR alpha: PPAR alpha fails to induce peroxisome proliferation associated genes in human cells independently of receptor levels. *J. Biol. Chem.* **276**, 1521-32527.

Lee, S. S., Pineau, T., Drago, J., Lee, E. J., Owens, J. W., Kroetz, D. L., Fernandez-Salguero, P. M., Westphal, H., Gonzalez, F. J. (1995). Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol Cell Biol*. **15**, 3012-3022.

Maloney, E. K., and Waxman, D. J. (1999). Trans-activation of PPARalpha and PPARgamma by structurally diverse environmental chemicals. *Toxicol Appl Pharmacol* **161:** 209-218.

Mukherjee R, Sun S, Santomenna L, Miao B, Walton H, Liao B, Locke K, Zhang J-H, Nguyen SH, Zhang LT, Murphy K, Ross HO, Xia MX, Teleha C, Chen S-Y, Selling B, Wynn R, Burn T, Young PR. (2002). Ligand and coactovator recruitment preferences of peroxisome proliferator activated receptor α. *J Steroid Biochem Mol Biol* **81:**217-225.

Osterburg, I. (1992). Diclofop-methyl; substance technical (code HOE 023408 OH ZD93 002) Two generation oral (dietary administration):reproduction study in the rat: (one litter per generation): Revised Final Report: Lab Project Number: A48800: HLD 169-023. Hazelton Deutschland GmbH, MRID 42543101, unpublished.

Palmer, C. N., Hsu, M. H., Griffin, K. J., Raucy, J. L., Johnson, E. F. (1998). Peroxisome proliferator activated receptor-alpha expression in human liver. *Mol. Pharmacol.* **53**, 14-22.

Peters, J. M., Cattley, R. C., Gonzalez, F. J. (1997). Role of PPAR alpha in the mechanism of action of the nongenotoxic carcinogen and peroxisome proliferator WY 14,643. *Carcinogenesis*. **18**, 2029-2033.

Pineau, T., Hudgins, W. R., Liu, L., Chen, L. C., Sher, T., Gonzalez, F. J., and Samid, D. (1996). Activation of a human peroxisome proliferator-activated receptor by the antitumor agent phenylacetate and its analogs. *Biochem Pharmacol* **52:** 659-667.

Pugh, G. Jr., Isenberg, J. S., Kamendulis, L. M., Ackley, D. C., Clare, L. J., Brown, R., Lignton, A. W., Smith, J. H., Klaunig, J. E. (2000). Effects of di-isononyl phthalate, di-2-ethylhexyl phthalate, and clofibrate in cynomolgus monkeys. *Toxicol. Sci.* **56**, 181-188.

Qualls CW, Hoivik DJ, Santostefano MJ, Brown HR, Anderson SP, Ott RJ, Oliver BR, Mudd PN, Mirabile RC, Kimbrough CL, Millet RT. 2003. Fibrates induce peroxisomal and mitochondrial proliferaion in cynomolgus monkeys without casusing cell cycle alterations or oxidative stress. Toxicologist 72 (S-1); 198. Abstract #961.

Rinde, E., Hill, R., Chiu, A., and Haberman, B. (1986). Proliferative hepatocellular lesions of the rat: review and future use in risk assessment. US EPA Risk Assessment Forum, Washington, DC.

Roberts, R.A., James, N.H., Woodyatt, N.J., Macdonals, N., Tugwood, J.D. (1998). Evidence for the suppression of apoptosis by the peroxisome proliferator activated receptor alpha (PPARα). *Carcinogenesis* **19(1):**43-48.

Roberts, R.A., James, N.H., Hasmall, S.C., Holden, P.R., Lambe, K., Macdonald, N., West, D., Woodyatt, N.J., Whitcome, D. (2000). Apoptosis and proliferation in nongenotoxic carcinogenesis: species differences and role of PPARα. *Toxicol. Lett.* 112-113;49-57.

Rodriguez, C., Noe, V., Cabrero, A., Ciudad, C. J., Laguana, J. C. (2000). Differences in the formation of PPARalpha-RXR/acoPPRE complexes between responsive and nonresponsive species upon fibrate administration. *Mol. Pharmacol.* **58**, 185-193.

Roglans, N., Bellido, A., Rodriguez, C., Cabrero, A., Novell, F., Ros, E., Zambon, D., Laguna, J.C. (2002). Fibrate treatment does not modify the expression of acyl coenzyme A oxidase in human liver. *Clin. Pharmacol. Ther.* **72(6):** 692-701.

Sapone, A., Peters, J.M., Sakai, S., Tomita, S., Papiha, S.S., Dai, R., Friedman, F.K., Gonzalez, F.J. (2000). The human peroxisome proliferator-activated receptor alpha gene: identification and functional characterization of two natural allelic variants. *Pharmacogenetics* **10(4):**321-333.

Schroeder, R.E., and Daly, I.W. (1983). A two-generation study with PPG-8, Project No. 82-2638, Accession Nos. 072201, 072202, 072203, Biodynamics, New Jersey ,unpublished.

Sher, T., Yi, H. F., McBride, O. W., Gonzalez, F. J. (1993). cDNA cloning, chromosomal mapping, and functional characterization of the human peroxisome proliferator activated receptor. *Biochemistry*. **32**, 5598-5604.

Shirasu, Y. (1987a). *Oxadiazon - 24 Month Chronic Toxicity and Carcinogenicity Study in Wistar Rats*. Unpublished report, MRID 40993401. Institute of Environmental Toxicology, Tokoyo, Japan.

Singh, I. and Lazo, O. (1992). Peroxisomal enzyme activities in brain and liver of pups of lactating mothers treated with ciprofibrate. *Neurosci. Lett*. **138(2):**283-286.

Sonich-Mullin, C., Fielder, R., Wiltse, J., Baetcke, K., Dempsey, J., Fenner-Crisp, P., Grant, D., Hartley, M., Knapp, A., Kroese, D., Mangelsdorf, I., Meek, E., Rice, J.M., and Younes, M. (2001) IPCS conceptual framework for evaluating a mode of action for chemical carcinogenesis. Regulatory Toxicology and Pharmacology **34:**146-152.

Staubli, W., Schweizer, W., Suter, J., and Weibel, E.R. (1977) The proliferative response of hepatic peroxisomes of neonatal rats to treatment with SU-13437 (nafenopin). *J. Cell Biol.* **74:**665-689.

Stefanini, S., Nardacci, R., Farioli-Vecchioli, S., Pajalunga, D., and Sartori, C. (1999) Liver and kidney peroxisomes in lactating rats and their pups after treatment with ciprofibrate. Biochemical and morphometric analysis. *Cell. Mol. Biol. (Noisy-le-grand)* **45(6):**815-29.

Stefanini, S., Serafini, B., Nardacci, R., Vecchioli, S.F., Moreno, S., and Sartori, C. (1995) Morphometric analysis of liver and kidney peroxisomes in lactating rats and their pups after treatment with the peroxisomal proliferator Di-(2-ethylhexyl)phthalate. *Biol. Cell* **85:**167-176.

Stefanini, S., Farrace, M.G., and Ceruargento, M.P. (1985). Differentiation of liver peroxisomes in the foetal and newborn rat. Cytochemistry of catalase and D-amino acid oxidase. *J. Embryol Exp Morphol* **88**:151-163.

Stefanini, S., Mauriello, S., Farrace, M.G., Cibelli, A., and Ceru, M.P.(1989) Proliferative response of foetal liver peroxisomes to clofibrate treatment of pregnant rats. A quantitative evaluation. *Biol. Cell* **67:**299-305

Tsukada, H., Mochizuki, Y., and Konishi, T. (1968) Morphogenesis and development of microbodies of hepatocytes of rats during pre- and postnatal growth. *J. Cell Biol.* **37**:231-243.

Tucker, M. J., and Orton, T. C. (1995). *Comparative Toxicology of Hypolipidaemic Fibrates*. Taylor and Francis, Bristol, PA.

Tugwood, J. D., Aldridge, T. C., Lambe, K. G., Macdonald, N., Woodyatt, N. J. (1996). Peroxisome proliferator-activated receptors: structures and function. *Ann. NY Acad. Sci.* **804**, 252-265.

USEPA (1986) Guidelines for Carcinogen Risk Assessment. Federal Register 51(185):33992-34003.

USEPA (1991). Alpha$_{2u}$-globulin: Association with chemically induced renal toxicity and neoplasia in the male rat. Risk Assessment Forum, Washington, DC.

USEPA (1996). Proposed Guidelines for Carcinogen Risk Assessment. Risk Assessment Forum, Washington, DC.

USEPA (1998). Assessment of follicular thyroid cell tumors. Risk Assessment Forum,Washington, DC.

USEPA (1999). Draft Revised Guidelines for Carcinogen Risk Assessment. Risk Assessment Forum, Washington, DC.

USEPA (2003). Draft final guidelines for carcinogen risk assessment. Risk Assessment Forum, Washington, DC.

Varanasi, U., Chu, R., Huang, Q., Castellon, R., Yeldandi, A. V., Reddy, J. K. (1996). Identification of a peroxisome proliferator-responsive element upstream of the human peroxisomal fatty acyl coenzyme A oxidase gene. *J. Biol. Chem.* **271**, 2147-2155.

Vohl, M.C., Lepage, P., Gaudet, D., Brewer, C.G., Bétard, C., Perron, P., Houde, G., Cellier, C., Faith, J.M., Després, J.P., Morgan, K., Hudson, T.J. (2000). Molecular scanning of the human PPARa gene: association of the L162V mutation with hyperapobetalipoproteinemia. *J. Lip. Res.* **41:**945-952.

Ward JM, Peters JM, Perella CM, Gonzalez FJ. (1998) Receptor and non-receptor mediated organ- specific toxicity Of di(2-ethylhexyl) phthalate (DEHP) in peroxisome proliferator- activated receptor a-null mice. *Toxicol. Path.* **26**, 240-246.

Wiebel, E.R., Staubli, W., Gnagi, H.R., and Hess, F.A. (1969). Correlated morphometric and biochemical studies on the liver cell. I. Morphometric model, stereologic methods, and normal morphometric data for rat liver. *J.Cell Biol.* **42:**68-91.

Wilson, G.N., King, T., Argyle, J.C. and Garcia, R.F. (1991). Maternal clofibrate administration amplifies fetal peroxisomes. *Pediatr. Res.* **29:**256-262.

Woodyatt, N. J., Lambe, K. G., Myers, K. A., Tugwood, J. D., Roberts, R. A. (1999). The peroxisome proliferator (PP) response element upstream of the human acyl CoA oxidase gene is inactive among a sample human population: significance for species differences in response to PPs. *Carcinogenesis*. **20**, 369-372.

Yamoto, T., Ohashi, Y., Furukawa, T., Teranishi, M., Manabe, S., and Makita, T. (1996). Change of the sex-dependent response to clofibrate in F344 rat liver during postnatal development. *Toxicol. Lett.* **85:**77-83.

Yu, S., Cao, W. Q., Kashireddy, P., Meyer, K., Jia, Y., Hughes, D. E., Tan, Y., Feng, J., Yeldandi, A. V., Rao, M. S., Costa, R. H., Gonzalez, F. J., Reddy, J. K. (2001). Human peroxisome proliferator-activated receptor {alpha} (PPAR{alpha})supports the induction of peroxisome proliferation in PPAR {alpha}deficient mouse liver. *J. Biol. Chem.* **276**, 42485-42491.

APPENDIX

A-1. PPARα Activity and Peroxisome Assemblage/Content in Fetal Liver Tissue

PPARα activity is expressed late in rodent embryonic development. In a study using *in situ* hybridization with antisense and sense riboprobes, expression of PPAR-α in E8.5 (gestation day 8.5), E11.5, E13.5, E15.5, and E18.5 Sprague-Dawley rat embryos was determined (Braissant and Wahli 1998). Low levels of transcripts of the PPARα gene were first detected in the livers of E13.5 embryos and became highly expressed in E18.5 embryos.

Wilson *et al.*, (1991) assayed liver tissue of gestational day 13 to 19 Swiss-Webster fetal mice for peroxisomal matrix, membrane-associated, and integral membrane proteins. Markers for these proteins used in the study were: matrix - catalase (not specific to peroxisomes but also found in the cytoplasm); membrane-associated protein - dihydroxyacetone phosphate acyltransferase (DHAP-AT); and integral membrane protein - peroxisomal membrane protein (PMP 70). DHAP-AT, which provides evidence of peroxisome assemblage during ontogeny, increased about 2-fold in fetuses from gestational day 13 to 19. There were low levels of PMP 70 in 13 - and 15-day fetuses; levels of this peroxisomal membrane protein were increased somewhat in 15- and 19-day fetuses. These data, and the fact that the numerical density of peroxisomes could not be quantified in fetal liver tissue due to weak staining of the peroxisomes indicate that the assemblage of peroxisomal proteins to form plasmids has not been completed in the gestational day 19 mouse fetus.

Catalase and palmitoyl CoA oxidase activities were first detected in the gestational day 15 Wistar rat fetus (Cibelli, *et al.*, 1988). In the liver, there was about a 2-3 fold increase in catalase activity and palmitoyl CoA oxidase activity between gestational days 19 and birth. Though these enzymes are not the products of PPARα target genes, they are indicative of peroxisome proliferation and therefore useful in establishing the “competency” of these test animals to respond to PPARα agonist exposure.

Peroxisomes are present (few to many) in gestational day 19 rat fetuses, few are present in gestational day 15 fetuses, and peroxisomes are not evident in fetal liver tissue before gestational days 14-15 (Tsukada, *et al.*, 1968; Stefanini *et al.*, 1985; Stefanini *et al.*,,1989; Cibelli *et al.*, 1988). In mice, few peroxisomes are found in 15-day fetal livers (Wilson *et al.*, 1991).

Table 1. Summary of Data on Peroxisome Assemblage/Content and PPARα Activity in Fetal Liver Tissue

Parameter	Indicator	Reference
Assemblage (membrane proteins)	Swiss-Webster mice - DHAP-AT - levels increased from gestational days 13-19; PMP 70 - low levels in gestational day 13 and 15 fetuses and increased slightly in gestational day 17 to 19 fetuses.	Wilson *et al.*, 1991.
Peroxisomal content	Few to many in gestational day 19 rat embryos but not evident before gestational days 14-15. Mean number in thin section of cell increases from 2.5 to 7.8 between 16 and 21 days of development; 7.8 in newborn and 30.1 in adult. Few found in gestational day 15 mouse embryos; inability to quantify the numerical density in gestational day 19 mouse embryos suggests assemblage of peroxisomal proteins into plasmids has not been completed.	Tsukada, *et al.*, 1968; Stefanini *et al.*, 1989; Cibelli *et al.*, 1988 Stefanini *et. al.*, 1985 Wilson, *et al.*, 1991
PPARα gene or peroxisomal activity	Low levels of PPARα gene transcripts first detected in gestational day 13.5 SD-rat embyros; highly expressed in gestational day 18.5 embryos Catalase and palmitoyl CoA oxidase activities first detected in gestational day 15 embryos and increase 2- to 3-fold at birth.	Braissant and Wahli, 1998 Cibelli, *et al.*, 1988

A-2. Peroxisome Assemblage/Content and Peroxisomal Enzyme Activity in Neonatal Liver Tissue

The activities of DHAP-AT and alkyl DHAP synthetase, enzymes involved in the synthesis of plasmalogens and that are found in peroxisomes, were reported to be similar in 19-day Sprague-Dawley neonates and adult rats (Singh and Lazo, 1992).

Stefanini *et al.*, (1995) found no statistically significant differences in numerical density or volume density of peroxisomes in livers of 14, 21, or 35-day F344 rat neonates and no differences between neonate groups and adult females. In a more recent report (Stefanini *et. al.*, 1999) no differences were found between 14 or 21-day neonates or between these groups and adult F344 rats in peroxisomal β-oxidation; the numerical density and volume density of liver peroxisomes were also comparable among groups. However, from a qualitative standpoint, hepatic peroxisomes were reported to be more numerous and larger in adult females than in neonates. There were gradual increases in catalase and palmitoyl CoA oxidase activities among the age groups (14-day neonate < 21-day neonate < adult). No differences were reported for the specific volume density among 7- 8.5-10- 13- or 17-day Wistar-derived rat neonates or among these neonates or when compared with adult data from a separate report (Staubli, *et al.*, 1977; Weibel, *et al.*, 1969).

There is evidence from other reports that peroxisomal enzyme activities that are markers of PPARα expression (e.g., palmitoyl CoA oxidase and carnitine acetyl transferase) are similar among control neonatal rats of different ages and neonatal and adult rats. As shown by Dostal *et al.*(1987), untreated Sprague-Dawley neonatal or young adult rats (10, 18, 25 or 46 days of age) were found to have levels of palmitoyl CoA oxidase activity (range - 3.61 -5.31 nmol/min/mg protein in neonates and young adults) comparable to adult rats 90 days of age (4.8 nmol/min/mg protein). Carnitine acetyl transferase activity was also shown to be comparable among neonates of different ages and similar to adults; carnitine acetyl transferase activity was: 6.97 $\pm$ 0.06, 5.85 $\pm$ 0.41, 11.4 $\pm$ 1.0, 8.10 $\pm$ 0.55, 6.18 $\pm$ 0.41, and 4.98 $\pm$ 0.63 nmol/min/mg protein for 10, 18, 20, 25, 46, and 90 day rats, respectively. Stefanini *et. al.*,(1999) found no differences between 14 or 21-day neonatal F344 rats or between these age groups and adult rats in peroxisomal β-oxidation but during development there were increases in catalase (14-day neonate about 5-fold less than the adult) and palmitoyl CoA oxidase (14-day neonate about 2-fold less than the adult) activities. In another study using F344 rats (Cimini *et al.*, 1994), palmitoyl CoA oxidase activity was found to be minimal in 14-day neonates (0.2 $\pm$ 0.02 mU/mg protein) when compared with enzyme activity in adults (3.7 $\pm$ 0.30 mU/mg protein. DHAP-AT was reported to be similar in 14-day neonates and adults and catalase enzyme activity increased about 3-fold between 14 days and adulthood.

Table 2. Ontogeny of Peroxisome Assemblage/Content and PPARα Activity in Neonatal Liver Tissue

Parameter	Indicator	Reference
Assemblage	DHAP-AT and alkyl DHAP synthetase activities similar in day 19 neonatal and adult rats. No difference in DHAP-AT activity between 14-day neonates and adults	Singh and Lazo, 1992 Cimini *et al.*, 1994
Peroxisomal content	No differences in numerical density or volume of peroxisomes among 14-, 21-, or 35-day or among these age groups and adult F344 rats. Qualitatively, peroxisomes more numerous and larger in adults. No differences in specific volume density of peroxisomes among Wistar-derived 7-, 8.5-, 10-, 13-, or 17-day rat neonates or between these age-groups and adults	Stefanini, *et al.*, 1995; Stefanini, *et al.*, 1999 Staubli, *et al.*, 1977; Weibel, *et al.*, 1969
Peroxisomal activity	No differences in peroxisomal β-oxidation among 14 day, 21 day, and adult F344 rats; palmitoyl CoA oxidase 2-fold less in 14 day neonates than in adults. Palmitoyl CoA oxidase activity comparable among 10 day, 18 day, 25 day, 46 day and adult SD rats; carnitine acetyl transferase activity similar among different age groups. Palmitoyl CoA oxidase activity minimal (about 19-30-fold less than adult activity) in 14-day F344 neonatal rats. Catalase activity (not specific to peroxisomes) 3-fold less in 14-day neonates than in adults.	Stefanini, *et al.*, 1999 Dostal, *et al.*, 1987 Cimini, *et al.*, 1994

A-3. Response of the fetal liver to the PPARα agonist clofibrate

Several studies have shown that treatment of pregnant rats and mice with the PPARα agonist, clofibrate, can induce peroxisome proliferation in fetal liver tissue.

Cibelli et al (1988) administered clofibrate (0.8% in the diet) to Wistar rats at various stages of pregnancy for 7 days. On the eighth day, dams were sacrificed, and the maternal and fetal livers were removed; some dams were allowed to deliver for examination of newborn livers. Liver weights were comparable among treated and control dams and among treated and control fetuses. There was a qualitative increase, relative to unexposed fetuses, in the size of peroxisomes in 15-day fetuses from treated dams and many peroxisomes were observed in 19-day fetuses and the newborn. There was no effect on catalase activity in 15- or 17-day embryos but there was a 3-fold increase in catalase activity in the newborn. Palmitoyl CoA oxidase activity was increased 4-fold in 15-day embryos and 6-8 fold in 19- to 21-day embryos. By birth, palmitoyl CoA oxidase activity was similar in the livers of the treated pups and dams. In the dams, palmitoyl CoA oxidase activity increased 3- to 4-fold and catalase activity increased 1.6- to 1.8-fold during days 15-17 of gestation, and during gestation days 17 to 21, palmitoyl CoA oxidase activity increased 4- to 5-fold and catalase activity increased 1.4- to 1.6 fold.

Stefanini *et. al.* (1989) treated pregnant Wistar rats with a diet containing 0.8% clofibrate for 7 days, and dams at 13, 15, 17, 19 and 21 days of pregnancy were sacrificed. Delivery was induced in dams close to term. Livers were removed from the

fetuses and newborn rats and morphometric analyses were conducted on hepatocyte peroxisomes. The volume density of peroxisomes and the peroxisomal numerical density were significantly increased in all test animals but particularly in fetuses over 19 days and the newborn. Increases in the numerical densities of 15-, 17-, 19-, 21- day fetuses and the newborn, relative to controls, were 1.5-, 1.3-, 1.8-, 2.5-, and 2.4-fold, respectively. Increases in the volume density of peroxisomes for these same age groups were 3.4-, 3.3-, 3.9-, 4.3-, and 4.6-fold, respectively.

Wilson *et al.* (1991) treated pregnant Swiss-Webster mice with 400 mg/kg/day clofibrate by gavage from gestation days 6-19. Peroxisomal density was increased 2-fold in maternal mice after 7 days of treatment. There was also an increase in the number of peroxisomes in 15-day fetuses from treated dams versus untreated dams but because of poor staining the peroxisomal density could not be quantified in the fetuses from treated dams. Maximal increases in peroxisomal membrane protein 70 were also observed 7 days after initiation of treatment in maternal liver tissue and general increases in peroxisomal proteins were observed in fetal liver tissues from clofibrate treated dams between 13 and 19 days of gestation. At gestation day 19, DHAP-AT and PMP 70 were each increased about 2-fold in clofibrate treated dams; DHAP-AT was also increased about 2-fold in gestation day 19 fetuses but PMP-70 was increased about 5-fold. Catalase activity in liver tissue of gestational day 19 fetuses was increased somewhat (1.2-fold to 1.8-fold) relative to catalase activity in fetuses from untreated dams but there was no effect on catalase activity in treated dams. Because catalase is found in the cytoplasm as well in peroxisomes, changes in catalase activity can not be ascribed solely to the effects on peroxisomes.

Table 3. Fetal Responses to the PPARα Agonist, Clofibrate.

Treatment	Fetal Response (compared to control)	Dams Response (compared to control)	Reference
Pregnant Wistar rats treated with 0.8% dietary supplement for 1 week prior to sacrifice	gestational day 15 - **palmitoyl CoA oxidase** activity increased 4-fold; **catalase** activity not affected; gestational days 19-21 - **palmitoyl CoA oxidase** activity increased 6- to 8-fold; at birth, specific activity about equal to treated dams **catalase** activity increased 3-fold in the newborn; specific activity less than treated dams **peroxisomes** - few in gestational day 15 fetuses, many in gestational day 19 fetuses and newborn, relative to controls **liver weights** - no increases during any stage of development	gestational day 15 - **palmitoyl CoA oxidase** activity increased 3.1-fold; **catalase** activity increased 1.8-fold gestational days 19-21 - **palmitoyl CoA oxidase** activity increased 5-fold; **catalase** activity increased 1.4- to 1.6-fold **peroxisomes** - not examined in dams **liver weights** - no statistically significant increases during gestation	Cibelli, *et al.*, 1988
Pregnant Wistar rats treated with 0.8% dietary supplement for 1 week prior to sacrifice.	**Numerical density of peroxisomes** - increased 1.3- to 2.5-fold in gestational day 15, 17, 19, 21 fetuses or newborn; **Volume density of peroxisomes** - increased 3.4- to 4.6-fold in gestational day 15, 17, 19, 21 fetuses or newborn	Not examined	Stefanini *et al.*, 1989

Treatment	Fetal Response (compared to control)	Dams Response (compared to control)	
400 mg/kg administered by gavage to pregnant Swiss-Webster mice from gestation day 6 to gestation day 19. Dams were sacrificed at 13, 15, 17 and 19 days of gestation, and maternal and fetal livers were removed.	**Peroxisomal density** could not be quantified in fetuses; general increases in peroxisomal proteins in gestational days 14-19 fetuses; **PMP 70** increased >5-fold in gestation days 14-19 fetuses; no effect in gestational day 13 or 15 fetuses; specific activity in 17- or 19-day fetuses about 4-fold less than that in treated dams **DHAP-AT** increased about 2-fold in gestational day 14-19 fetuses; minimal effect on gestational day 15 fetuses; no effect on gestational day 13 fetuses; specific activity about 50-fold less in gestational day 17 or 19 fetuses than in treated dams **Catalase** - 1.2- to 1.8-fold increase in gestational day 19 fetuses; no effect in gestational day 13, 15, or 17 gestational day fetsuses	**Peroxisomal density** increased 2-fold after 7 days treatment; **PMP-70** and **DHAP-AT** each increased about 2-fold **Catalase -** unaffected	Wilson *et al.*, 1991

A-4. Response of Neonates Following Lactational Exposures to PPARα Agonists

Peroxisomal enzyme activities were increased in nursing pups from Sprague-Dawley rat dams that were treated with 0.025% ciprofibrate in the diet from postnatal days 3-19 (Singh and Lazo, 1992). DHAP-AT and alkyl-DHAP synthetase activities were increased in the livers of 19-day neonates by 3.9 and 2.6-fold, respectively; corresponding increases in activities of these two enzymes were also seen in the ciprofibrate-treated dams (4.2 and 3.2-fold, respectively). Liver weights of treated dams were increased 1.8-fold and in 19-day pups liver weights were increased 1.5-fold.

Cimini *et al.* (1994) treated F344 damswith 1g/kg/day DEHP by gavage for up to 21 days from day of delivery through lactation. Pups were sacrificed on day 14, day 21 or day 35 following 14 days of recovery. Relative liver weights increased 1.65-fold in the dams at weaning, and 1.47-fold in 14- and 21-day pups. At day 21, palmitoyl CoA oxidase increased 9.3 fold in dams, while it increased 6-fold in the nursing pups at 14 days and 4.85-fold at 21 days. However, palmitoyl CoA oxidase activity was substantially less in the pups than in the dams treated with DEHP (pups, 1.2 mU/mg protein at 14 days; dams, 34.4 mU/mg protein at 21 days). DHAP-AT was increased about 2-fold in 14- and 21-day neonates, but DHAP AT levels were unaffected in DEHP-treated dams. Catalase activity was increased about 2-fold in 14-day and 21-day neonates and adults. Following 14 days of recovery, most enzyme levels returned to normal in the dams and pups, although catalase activity remained slightly higher in both the dams and pups.

In a separate study, pregnant lactating F344 dams were administered by gavage 1 g/kg/day DEHP for 21 days beginning at the day of delivery and the nursed pups were sacrificed after 2 or 3 weeks, or following a 14 day recovery period (Stefanini, *et al.*, 1995). The numerical density or volume density of peroxisomes was increased marginally (< 2-fold), relative to controls, in both pup groups. Dams treated for 21 days with DEHP showed a more pronounced increase in the volume density of peroxisomes (about 2-fold), but the numerical density of peroxisomes was increased in the dams to the same degree as the 2- or 3-week pups. The increases in volume density or numerical density of peroxisomes did not decline to control levels in the 3-week pups after a 14 day recovery period. Volume density of peroxisomes apparently declined to close to control levels after a recovery period of 8 days in dams treated for three-weeks but there was no apparent decline in the numerical density of peroxisomes. Relative liver weights were increased about equally in 2 and 3 week-old neonates and adults (1.5 to 1.6-fold).

Neonatal F344 rats exposed for 14 days following birth to nafenopin (NF) or Wy-14,643 (WY) through milk showed increases in peroxisomes and peroxisomal enzyme activities (Fahl, 1983). The dams were treated twice daily by gavage with 100 mg/kg NF or WY. Increases in peroxisome numbers were comparable in lactating dams treated with nafenopin and their suckling offspring. In pups exposed lactationally to NF for14 days, there was a 3-fold, 35-fold, 29-fold and 14-fold increase in the activities of catalase. carnitine acetyl transferase, peroxisomal enoyl CoA hydratase, and palmitoyl

CoA oxidase, respectively; exposure to WY for 14 days resulted in a 3-fold, 15-fold, 46-fold, and 12-fold increase in the activities of catalase. carnitine acetyl transferase, peroxisomal enoyl CoA hydratase, and palmitoyl CoA oxidase, respectively. The increases in peroxisomal enzyme activities catalase and peroxisomal enoyl-CoA hydratase were similar to those seen in the dams treated with the PPARα agonists for 14 days.

Effects of treatment with ciprofibrate on PPARα expression were also investigated in lactating F344 rats and their pups (Stefanini, *et al.*, 1999). Dams were treated with ciprofibrate (0.025% in the diet) beginning on the day of delivery for 21 days or a week after delivery for 14 days (i.e., days 7-21 after delivery). Pups from dams treated with ciprofibrate for 21 days were sacrificed at 14 or 21 days; pups from dams treated with ciprofibrate from day 7 to day 21 were sacrificed at 21 days. Palmitoy CoA oxidase activity was increased 12-14-fold, relative to controls, in pups from dams treated with ciprofibrate from either day 7 to day 21 (14 days) or for 21 days; the levels of activity (approximately 4 U/g protein) were comparable to adults treated with ciprofibrate for 21 days . However, in the 7-21-day pups, greater increases in enzyme activity, relative to controls, were seen than in adults treated with ciprofibrate for 14 days and the level of enzyme activity in the pups exposed lactationally to ciprofibrate was also greater (about 2X) than the treated dams (7-21-day pups - about 4 U/g protein or a 12-fold increase relative to controls; adults - about 2 U/mg protein or a 5-fold increase relative to controls). Increases in cyanide insensitive peroxisomal β-oxidation activity, up to 10-fold, relative to controls, were seen in 14-day pups from treated dams and in day 7 to day 21 pups. However, the induced levels of β-oxidation (U/g of liver tissue) in day 1-14 pups and in day 7-21 pups were less than the induced levels of β-oxidation seen in dams treated with ciprofibrate for 14 days. The levels of induced β-oxidation in 21-day pups from dams treated with ciprofibrate for 21 days were comparable. There was about a 3 to 4-fold increase, relative to controls, in numerical density of liver peroxisomes in day 1-14 pups and in day 7-21 pups. The increase in this parameter in dams treated with ciprofibrate for either 14 days or for 21 days was about 2-fold. Finally, increases in liver weights were somewhat more pronounced in the pups lactationally exposed to DEHP than in treated dams (relative liver weight increases - day 1-14 pups, 2.25X and day 7-21 adults, 1.47X; day 7-21 pups, 2.63X and day 7-21 adults, 1.47X; day 1-21 pups, 2.63X and day 1-21 adults, 1.94X.

In a two-generation rat reproduction study with diclofop-methyl submitted to the Agency (Osterburg, 1992), increased liver weights were reported at 100 ppm (the highest dose tested) in F0 and F1 adults (males 12% and 31%; females 26% and 13%), no increases in liver weights were reported for F1 male or female day 4 offspring but increased liver weights were reported for F2 male day 4 offspring (24%) and F2 female day 4 offspring(23%). At the highest dose tested, histologic examination showed that there was nuclear swelling of hepatocytes and hepatocyte hypertrophy in both F0 and F1 adults. No hepatic effects were observed in F1 offspring but cellular hypertrophy and nuclear swelling was observed in F2 offspring. Thus, the results of the study with diclofop-methyl show that, qualitatively, exposure of dams to a PPARα agonist can lead to effects on liver weights and liver histology in neonates that are consistent with PPARα agonism.

In contrast, a two-generation reproduction study with lactofen submitted to the Agency showed no effects on liver weights or liver histology in neonates(Schroeder, 1983). In this study, liver weights were increased somewhat (113%) in F1 adult females treated with 2000ppm but not in F1 or F2 weanlings. No liver histopathology was observed in F0 adult males (histopathology was not conducted on female F0 adults) but intrahepatic bile duct proliferation, centrolobular degeneration/necrosis was observed in male and female rats dying on test. Histopathology examination of liver tissue from F1 or F2 weanlings revealed no histopathologic effects.

The results of these two studies show that when dams are treated with a PPARα agonist (i.e., diclofop-methyl) during gestation and lactation, liver hypertrophy may be induced in the nursing neonate. However, the liver effects of the PPARα agonist in neonates are no more pronounced than those observed in adults (i.e., diclofop-methyl) or they are absent in the neonate but present in the adult (i.e., lactofen).

Table 4. PPARα Agonism in Dams and in Neonates Exposed by Lactation to Peroxisome Proliferators

Chemical	Treatment	Neonatal Response	Adult Response	Reference
Ciprofibrate	0.025% in the diet of SD rat dams from postnatal days 3-19	DHAP-AT increased 3.9-fold and alkyl-DHAP synthetase increased 2.6-fold in 19-day nursing neonates; Liver weights increased 1.8-fold	DHAP-AT increased 4.2-fold and alkyl-DHAP synthetase 3.2-fold at postnatal day 19 Liver weights increased 1.5-fold	Singh and Lazo, 1992
Ciprofibrate	F344 rat dams treated with 0.025% ciprofibrate in the diet from day of delivery to day 21 or a week after delivery for 14 days	palmitoyl CoA oxidase activity increased 12-14-fold in 21-day pups from dams treated for 21 days or 21-day pups from dams treated from postnatal days 7-21. peroxisomal β-oxidation increased up to 10-fold in day-14 pups and in day 21 pups from dams treated from day 7-21. numerical density of peroxisomes increased in 3- to 4-fold in 14-day pups and in day 21 pups from dams treated from day 7 - 21;	palmitoyl CoA oxidase activity increased 5-fold in dams treated for 14 days (day 7-21); peroxisomal β-oxidation increased 6-8-fold (estimated from fig. 1) in adults treated for 14 days or 21 days; numerical density of peroxisomes increased 2-fold in dams treated for 14 or 21 days.	Stefanini *et al.*, 1999
DEHP	F344 rat dams gavaged with 1g/kg/day from parturition to day 21.	at 14 or 21 days, palmitoyl CoA oxidase increased 4.85 to 6-fold; DHAP-AT increased about 2-fold; at 21 days and 14 days recovery, DHAP-AT remained increased (about 2-fold). catalase increased 2-fold. relative liver weights increased 1.47-fold in 14-day neonates and about the same amount in 21-day neonates	at 21 days: palmitoyl CoA oxidase increased 9-fold; DHAP-AT unchanged; at 21 days and 14 days recovery, DHAP-AT returned to control levels. catalase increased 2-fold. relative liver weights increased 1.65-fold	Cimini *et al.*, 1994
DEHP	F344 rat dams gavage with 1g/kg/day from parturition to day 21	numerical density and volume density of peroxisomes increased marginally (<2-fold) in 2- or 3-week nursing pups; volume or numerical density of peroxisomes in 3-week pups did not decline following a 14-day recovery period.	a more pronounced increase in volume density of peroxisomes (about 2-fold) than 2- or 3-week nursing pups; volume density, but not numerical density, of peroxisomes declined to control levels following a 8-day recovery in dams treated for 21 days.	Stefanini *et al.*, 1995

Chemical	Treatment	Neonatal Response	Adult Response	
Nafenopin (NF) or Wy-14,643 (Wy)	F344 rat dams gavaged with 100 mg/kg NF or Wy twice daily from delivery to postnatal day 14	catalase, NF and Wy - 3-fold increase at 14 days; carnitine acetyl transferase, NF 35-fold increase and Wy 15-fold increase; peroxisomal enoyl CoA hydratase, NF 29-fold increase and Wy 46-fold increase; palmitoyl-CoA oxidase, NF 14-fold increase and Wy 12-fold increase.	catalase and peroxisomal enoyl-CoA hydratase were similar to those seen in the day 14 neonates; peroxisome numbers were comparable, qualitatively, in lactating dams treated with nafenopin and their suckling offspring	Fahl *et al.*, 1983
Diclofop methyl	Sprague-Dawley male and female rats administered diclofop-methyl via the diet for two consecutive generations; dose levels - females 0, 0.9, 2.5, or 8.5 mg/kg/day	liver weights - no increases in F1 male or female day 4 offspring F2 males - increased 24%; F2 females - increased 23% no hepatic histopathology	liver weights - F0 males increased 12%; F1 males increased 31%; F0 females increased 26%; F1 females increased 13% nuclear swelling and hepatocyte hypertrophy in both F0 and F1 adults	Osterburg, 1992
Lactofen	Sprague-Dawley male and female rats administered lactofen via the diet for two generations; dose-levels - F0 females 0, 3.1, 31.8, or 121.3 mg/kg/day; F1 females 3.3, 32.9, or 121.3 mg/kg/day	liver weights - no increases in F0 or F1 pups; no hepatic histopathology observed in F0 or F1 offspring	liver weights - increased 13% in F1 adult females; centrolobular degeneration/necrosis was observed in male and female rats dying on test	Schroeder, 1983

A-5. Response of Neonates Following Direct Exposures to PPARα Agonists

A study designed to investigate the effects of a PPARα agonist on neonatal rats of different ages was conducted by Dostal *et al.* (1987). Male Sprague-Dawley rats 6, 14, 16, 21, 42, or 86 days of age were administered (by gavage) daily doses of DEHP for 5 days, and 24 hours after sacrifice activities of hepatic peroxisomal enzymes, palmitoyl CoA oxidase and carnitine acetyltranferase were determined. The doses administered were 0, 10, 100, 1000, or 2000 mg/kg/day. Administration of 1000 mg/kg/day caused significant decreases in body weight and mortality (66-70%) in pups 14-18 days of age, and administration of 2000 mg/kg/day caused mortality in virtually all pups of these ages (the authors concluded that body weight decrements and mortality were not associated with effects on peroxisome proliferation activity). At a non-lethal dose (100 mg/kg/day), absolute liver weight increases relative to those in the controls were 0, 17, 3, 10, and 14% (6-10, 14-18, 21-25, 42-46, and 86-89-day old pups and adults, respectively). At 100 mg/kg/day, measurements of palmitoyl CoA activity showed that there was a greater increase only in the 14-18-day pups when compared with 86-90-day adults (6.9-fold increase versus a 3.98-fold increase). A greater increase in carnitine acetyl transferase also was shown at this dose level only for 14-18 day pups when compared with 86-90-day adults (7.8-fold increase versus a 4.4-fold increase). The data on increased liver weights and peroxisomal enzyme activities from this study indicate that there is little difference in the response of neonatal or young adult rats compared with adult rats to treatment with DEHP.

Administration of clofibrate to 4-, 8-, or 12-week old male or female F344 rats for 7 days (200 mg/kg/day) induced increases in liver weights, peroxisomal β-oxidation, and the percentage of peroxisomal area relative to hepatocellular cytoplasm (Yamoto, 1996). Increases in these parameters among the 4-, 8, and 12-week old rats, respectively, were: relative liver weights - males - 108, 161, and 168%; females - 108, 119, and 117%; palmitoyl CoA oxidation - males - 206, 589, and 1072% and females - 145, 152, and 312%; percentage of peroxisomal area to hepatocellular cytoplasm - males - 134, 479, and 657% and females - 168, 236, 169%. All increases were statistically significant ($p < 0.05$). The results of this study indicate that the effects of the PPARα agonist clofibrate are weak in the immature rat and that susceptibility to the effects of the chemical increase as rats approach adulthood.

Peroxisome volume density was increased 6-fold and peroxisome number was increased 2-fold following treatment by intubation of neonatal Wistar-derived rats with 100 mg/kg/day nafenopin from lactation days 5 through 9 (Staubli, *et al.*, 1977). Following a recovery period of 7 days, peroxisome volume density and number closely approached, but did not attain, control values.

Table 5. Data on liver effects in Neonates or Weanlings Exposed Directly to PPARα agonists

Chemical	Treatment	Neonatal/Weanling Response	Adult Response	
DEHP	male Sprague-Dawley rats 6, 14, 16, 21, 42, or 86 days of age gavaged with 0, 100, 1000, or 2000 mg/kg/day for 5 days (NOTE: 1000 and 2000 mg/kg/day were lethal doses)	at dose = 100 mg/kg/day: Palmitoyl CoA oxidase increase 6-10 day-3X 14-18 day-7X 21-25day-2X carnitine acetyl transferase increase 6-10 day-2.7X 14-18 day-7.8X 21-25 day-2.4X increases in peroxisomes - similar at all ages (qualitatively) liver weight increases 14-18 day - 1.2X	at dose = 100 mg/kg/day: Palmitoyl CoA oxidase increase 42-46 day-2.5X 86-90 day-4X carnitine acetyl transferase increase 42-46 day-young adult - 3.6X 86-90 day adult - 4.4X increases in peroxisomes - similar at all ages (qualitatively) liver weight increases 42-46 day young adult - 1.1X 86-90 day adult - 1.1X	Dostal *et al.*, 1987
Nafenopin	5-day Wistar-derived rat pups intubated with 100 mg/kg/day from day 5 through day 9	peroxisome volume density increase - 6-fold in day 9 neonates peroxisome number increase - 2-fold in 9 day neonates	no data	Staubli, *et al.*, 1977

Chemical	Treatment	Neonatal/Weanling Response	Adult Response	
Clofibrate	4-, 8-, or 12-week male and female F344 rats gavaged with 200 mg/kg/day for 7 days.	palmitoyl CoA oxidase increase males 4-week weanling- 206 % 8-week young adult - 589% females 4-week weanling - 145% 8-week young adult - 152% percentage of peroxisomal area to cytoplasm - increase males 4-week weanling- 134% 8-week young adult- 479% females 4-week weanling- 168% 8-week young adult- 236% increased liver weights males 4-week weanling - 108% 8-week young adult - 161% females 4-week weanling - 108% 8-week young adult - 119%	palmitoyl CoA oxidase increase males 12-week adult- 1072% females 12-week adult - 312% percentage of peroxisomal area to cytoplasm - increase males 12-week adult- 657% females 12-week adult - 169% increased liver weights males 12-week adult - 168% females 12-week adult - 117%	Yamoto, 1996